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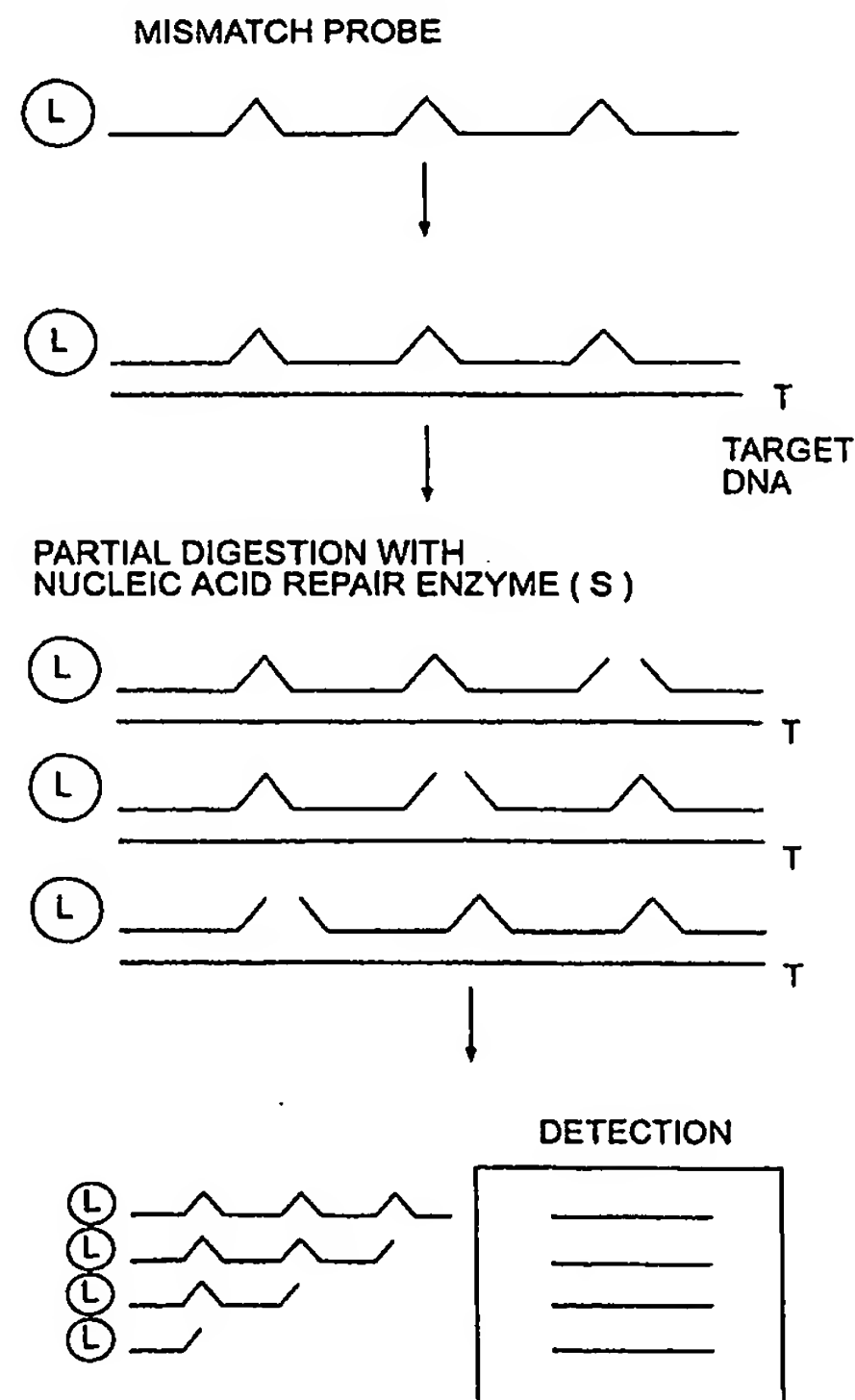
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(54) Title: DETECTION AND MAPPING OF POINT MUTATIONS USING PARTIAL DIGESTION

(57) Abstract

According to the present invention there is provided a method for detecting the presence of at least two point mutations in a target polynucleotide, as well as their relative positions and specific nucleotide positions, involving partial digestion and the use of mismatch repair enzymes.



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## DETECTION AND MAPPING OF POINT MUTATIONS USING PARTIAL DIGESTION

### FIELD OF THE INVENTION

The present invention relates to a method for detecting the presence of at least two point mutations in a target polynucleotide, as well as their relative positions and specific nucleotide position, via partial digestion. The present invention further relates to a method for detecting the presence of at least two point mutations in a target polynucleotide, as well as their relative positions and specific nucleotide position via a combination of partial digestion and an oscillation reaction.

### BACKGROUND OF THE INVENTION

Genomic DNA provides the template for the information that allows the generation of proteins which are expressed and made by an organism. These proteins are generally essential for the survival of any specific cell in an organism. Therefore, the organism requires the template to be correct and free of mistakes in order to generate a protein that is functional in a cell. The protein may be nonfunctional if a single nucleotide of this DNA sequence is mutated ("a point mutation"). Point mutations which elicit disease states are known for many proteins.

Recent advances have allowed for the detection of point mutations with mismatch repair enzymes. Hsu et al., *supra*, describe the detection of A/G point mutations with mutY repair enzyme. Xu et al., *Carcinogenesis* 17(2): 321 (1996) further describe using mutY to detect A/G and to a lesser extent C/A mutations.

Youil et al., *PNAS* 92: 87 (1995) relate techniques for screening for each of the possible eight point mutations, i.e. G/A, C/T, C/C, G/G, A/A, T/T, C/A, and G/T, using T4 endonuclease VII. Lu et al., WO93/20233 at 29-30 describe screening for mutations using all-type enzyme, which recognizes all eight base pair point mutations. Lu et al. also describe the use of combinations of different repair enzymes to ascertain the presence of an unknown point mutation in a sample. *Id.* at 27.

These techniques are directed, however, to the detection of a single point mutation in a given polynucleotide sample. To the extent that a nucleic acid target molecule has multiple base pair mutations spanning its length, the above methods are either ineffective or inefficient in detecting the presence and relative position of these mutations.

#### SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for detecting the presence of at least two point mutations in a target polynucleotide, as well as their relative positions and specific nucleotide position via partial digestion.

It is a further object of the present invention to provide a method for detecting the presence of at least two point mutations in a target polynucleotide, as well as their relative positions and specific nucleotide position via a combination of partial digestion and an oscillation reaction.

In accomplishing the foregoing objects as well as other objects, there is provided a method of detecting the presence of and determining the relative positions of at least two point mutations in target polynucleotides, comprising:

(a) hybridizing single-stranded oligonucleotide probes to target polynucleotides to form hybrid, double-stranded polynucleotides such that mismatches occur at

the sites of the point mutations, wherein the probes are complementary to a non-mutated sequence of the target polynucleotides and are labelled at one end but not both ends, and wherein the target polynucleotides are not labelled;

(b) partially digesting the probe strands of the hybrid polynucleotides with a nucleic acid repair enzyme such that probe fragments of differing lengths are generated;

(c) separating the probe fragments by size in a medium suitable for visualizing the separated probe fragments; and then

(d) visualizing the separated probe fragments in the medium, whereby the presence and relative positions of the point mutations are determined.

There is further provided a method of detecting the presence of and determining the relative positions of at least two point mutations in a target polynucleotide, comprising:

(a) hybridizing a single-stranded oligonucleotide probe to a target polynucleotide to form a hybrid, double-stranded polynucleotide such that mismatches occur at the sites of the point mutations, wherein the probe is complementary to a non-mutated sequence of the target polynucleotide and is labelled at one end but not both ends, and wherein the target polynucleotide is not labelled;

(b) partially digesting the probe strand of the hybrid polynucleotide with a nucleic acid repair enzyme producing oligonucleotide fragments, wherein the oligonucleotide probe is designed such that the oligonucleotide fragments dissociate from the target polynucleotide spontaneously at a predetermined temperature;

(c) repeating steps (a) and (b) such that probe fragments of differing lengths are generated;

(d) separating the probe fragments by size in a medium suitable for visualizing the separated probe fragments; and then

(e) visualizing the separated probe fragments in the medium, whereby the presence and relative positions of the point mutations are determined.

#### BRIEF DESCRIPTION OF THE DRAWINGS

5           FIGURE 1 is a schematic diagram showing the detection of three point mutations using the partial digestion method of the instant invention. Probe is hybridized to target and then nucleic acid repair enzyme is added, which partially digests the probe. The  
10           resulting probe fragments are then detected.

          FIGURE 2 is a schematic diagram showing the detection of two point mutations using partial digestion in combination with an oscillation reaction. Probe is hybridized to target and then nucleic acid repair enzyme  
15           is added, which partially digests the probe. The probe then dissociates from the target, with or without an increase in temperature, and the cycle is repeated.

          FIGURE 3 is a representation of the DNA sequence of *orf10* and the amino acid sequence of the corresponding  
20           ORF10 polypeptide.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

          The above-mentioned disadvantages of conventional techniques for detecting multiple point mutations have been overcome by the present invention. In particular,  
25           it has been discovered that partial digests, effected using mismatch repair enzymes, allow for the detection and mapping of multiple base pair mutations.

          Partial digest mapping has been limited heretofore to restriction endonucleases. For example, see Danna et  
30           al., 65 METHODS IN ENZYMOLOGY, Ch. 53, at page 449 (Academic Press 1980). Restriction endonucleases are not desirable for detecting multiple point mutations, however, because a restriction enzyme can only detect a



mutation if it is located within the enzyme's specific cleavage recognition site.

The instant invention therefore has overcome the above-mentioned disadvantages of existing techniques for detecting point mutations. The inventive approach entails hybridizing single-stranded oligonucleotide probes to target polynucleotides to form hybrid, double-stranded polynucleotides. The hybridization preferably occurs under conditions that are "stringent," which typically implicates conditions that include a 50-100 mM salt solution at a temperature of  $(3N - 20^{\circ}\text{C})$ , where N is the number of nucleotides in the oligonucleotide probe.

As for probe design, preferably, the oligonucleotide probe is designed not to have self complementary regions, palindromic regions and the probe must also have probe specificity. The parameters for probe design can be found in Lowe et al., *Nucl. Acids Res.* 18:1757-1761 (1990); Rychlik et al., *loc. cit.* 17:8543-8551 (1989); Rychlik et al., *loc. cit.* 18:6409-6412 (1990), which discusses probe design as applied to PCR reactions.

The probe may be a synthetic oligonucleotide, or may be derived from the PCR amplification of a desired genomic DNA or cDNA sequence by amplifying the desired sequence with two primers one of which is labelled. The probe may also be a restriction fragment generated from restriction endonuclease cleaving of a desired genomic DNA or cDNA sequence.

The oligonucleotide probe also is labelled. Probe labelling allows for the detection of cleaved oligonucleotide fragments and may be accomplished by a number of art recognized methods. For example, the oligonucleotide can be tagged with a radioactive label, such as a radiolabelled nucleotide. Alternatively, the probe can be labelled with biotinylated nucleotides or florescent nucleotides as known to those of skill in the art.

In the present invention, the oligonucleotide probe is labelled at one end but not both. Additionally, the target polynucleotide is not labelled.

Because the probe is complementary to a non-mutated sequence of the target polynucleotide, there will be mismatches between non-mutated probe and mutated target polynucleotides at each site of point mutation. In the  
5 instant invention, the target polynucleotide will have at least two mismatch sites.

The probe strands of the hybrid probe-target polynucleotides are then partially digested with a "nucleic acid repair enzyme," such that probe fragments  
10 of differing lengths are generated. The probe fragments are then separated by size in a medium suitable for visualizing the separated fragments. The separated fragments are then visualized, and the presence and relative position of the point mutations are determined.

15 In a further embodiment, the size of the separated probe fragments are measured and thereby the specific nucleotide position of each point mutation is determined. Methods for measuring the size of nucleotide bands in visualizing medium are known to those of skill in the  
20 art. For example, a size marker can be used to determine the size of a nucleotide band in an electrophoresis gel.

The phrase "polynucleotide target" is used here to denote a nucleotide sequence of any size, including a sequence of less than 30 base pairs, or up to several  
25 hundred base pairs. For instance, examples of polynucleotide targets include the genes or portions of genes which have been isolated from genomic or cDNA libraries by methods known to the skilled artisan, such as PCR amplification. In particular, genes of interest  
30 would be those known to exhibit point mutations eliciting disease states. Examples include sickle cell anemia hypoxanthine phosphotransferase and p53, a tumor suppressor gene, as well as several oncogenes and cancer genes. Other illustrations of polynucleotide targets  
35 include cDNAs generated by reverse transcriptase, and a specific deoxyoligonucleotide primer encompassing a short sequence immediately downstream of a hotspot for mutations within a gene such as BRCA1.



Other examples of polynucleotide targets include cDNAs prepared by reverse transcriptase using mRNA as template and a deoxynucleotide primer specific for the mRNA of interest. In this case the cDNA would act as a target for a labelled oligonucleotide to generate a base mismatch or several base mismatches upon hybridization. The addition of DNA mismatch repair enzymes would result in the formation of cleaved products whose intensity could allow for the quantitation of the mRNA or to determine if the genomic DNA encoding this mRNA has one or more point mutations.

In fact, this procedure can act as an endogenous method for amplifying a target gene, such as BRCA1, in that a cell's own production of mRNA produces a desired amount of target polynucleotide for a partial digestion assay. Detection of point mutations within target cDNAs by this embodiment can therefore obviate the need for amplification by PCR.

Another example of a polynucleotide target includes bacterial 16S rRNA genes and intergenic regions. See Brow et al., *J. Clin. Microbiol.* 34:3129-3137 (1996). In this scenario, a 350 bp segment of *E. coli* 16S rRNA DNA is amplified by PCR using a labelled primer and an unlabelled primer. The labelled PCR product, labelled at one end on one of the strands, is mixed with a target DNA from an unknown bacterium comprising a PCR product generated with the same unlabelled primers. Denaturation and renaturation of the target and probe DNAs generates a proportion of molecules in which the labelled strand comes from the *E. coli* and the other strand comes from the unknown bacterium. A number of base mismatches are created between the two strands which are cleaved by the DNA mismatch repair enzymes. The cleaved products are detected on a denaturing polyacrylamide gel or by other methods known to the skilled artisan. The pattern of the cleavage products would be characteristic of the sequence of the unknown bacterium's 16S rRNA gene and could therefore be a means of identification of the unknown organism.

In the present description, the phrase "nucleic acid repair enzyme" denotes an enzyme that cleaves, at a point of mismatch, one strand of a duplex formed by oligonucleotide probe and target polynucleotide. Examples of nucleic acid repair enzymes which can be used in the above process are mutY (Wu et al., *Proc. Nat'l Acad. Sci. USA* 89: 8779-83 (1992)), T/G mismatch-specific nicking enzyme from HeLa nuclear extracts (Wiebauer & Jiricny, *Nature* 339: 234-36 (1989); Wiebauer & Jiricny, *loc. cit.* 87: 5842-45 (1990)), T/G mismatch-specific nicking enzyme from *E. coli* (Hennecke et al., *Nature* 353: 776-78 (1991)), human yeast all-type enzymes (Yeh et al., *J. Biol. Chem.* 266: 6480-84 (1991); Chiang & Lu, *Nuc. Acids Res.*, 19:4761-4766 (1991)), Deoxyinosine 3'-Endonuclease from *E. coli* (Yao et al., *J. Biol. Chem.* 270: 28609-16 (1995); Yao et al., *J. Biol. Chem.* 269: 31390-96 (1994)).

Another example of nucleic acid repair enzyme is an enzyme system comprising a glycosylase combined with an AP cleaving enzyme, such as endonuclease or lyase. Together glycosylase and AP cleaving enzyme, such as endonuclease or lyase cleave oligonucleotide probe/target polynucleotide duplex at a point of mismatch. A glycosylase creates an abasic sugar (an AP site) at the point of mismatch, which then is cleaved by an AP cleaving enzyme, such as endonuclease or lyase. Illustrative enzymes in these categories are detailed below:

I. glycosylases -- tag-1, alkA, ung, fpy, mutY, nth, xthA, nfo, recJ, uvtA, uvrD, mfd, mutH, mutL, mutS, uracil DNA glycosylase, hydroxymethyluracil glycosylase, 5-mC DNA glycosylase, hypoxanthine DNA glycosylase, thymine mismatch DNA glycosylase, 3-mA DNA glycosylase, hydrated thymine DNA glycosylase (endonuclease III), pyrimidine dimer glycosylase. These enzymes can come from any different biological sources. For example, Friedberg et al., *DNA REPAIR AND MUTAGENESIS* (ASM Press 1995), lists uracil DNA glycosylases from herpes simplex

virus types 1 and 2, equine herpes virus, Varicella  
zoster virus, Epstein Barr virus, human cytomegalovirus,  
*Mycoplasma lactucae*, *E. coli*, *B. subtilis*, *M. luteus*, *B.*  
*steorophermaophilus*, *Thermothrix thirpara*, *S. pneumoniae*,  
5 *Dictyostelium discoideum*, *Artenia salina*, *S. cerevisiae*,  
*Hordeum vulgare*, *Zea mays*, *Triticum vulgare*, rat liver  
mitochondria, calf thymus, human placenta, HeLa S3 cells,  
and acute leukemia blast cells.

10 II. AP cleaving enzymes -- *E. coli* exonuclease III, *E.*  
*coli* endonuclease IV, *Saccharomyces* AP endonuclease,  
*Drosophila melanogaster* AP endonuclease I and II, human AP  
endonuclease, human AP lyase, BAP endonuclease, APEX  
endonuclease, HAP1 and AP endonuclease

15 In addition to the above systems, cleavage may also  
be effected by using a glycosylase enzyme, as described  
above, in combination with basic conditions and increased  
temperature. In this embodiment, increasing pH and  
temperature effectuates cleavage at the AP site created  
by the glycosylase enzyme. Suitable parameters for  
20 cleavage of the AP site are pH levels of approximately 8  
to 14, and temperatures ranging from approximately 50° to  
95°C.

In another embodiment, the present invention employs  
a nucleic acid repair enzyme that is thermally stable, in  
25 the sense that the enzyme would function at some elevated  
temperature, such as from 50° to 80°C. Additionally, it  
is preferable that the thermally stable nucleic acid  
repair enzyme withstand temperatures up to 100°C for  
short periods.

30 For instance, the present invention contemplates the  
use of a thermally stable glycosylase. An example of a  
thermally stable glycosylase is the ORF10 protein encoded  
by the DNA sequence of Figure 3. This enzyme has been  
synthesized by Richard P. Cunningham at the State  
35 University of New York at Albany, according to the  
methods of Example 4. See also Horst et al., *EMBO J.* 15:  
5459 (1996).

The substrate activity of the ORF10 enzyme includes both base cleaving properties and AP endonuclease activities. The AP endonuclease activities of this enzyme may be enhanced, however, by changing the amino acid residue in position 126 of Figure 1 from a tyrosine to a lysine. This substitution may be achieved by site directed mutagenesis by the methods discussed in Deng, et al., *J.A. Anal. Biochem.* 200:81 (1992).

The ORF10 glycosylase is a homologue of the endonuclease III family. As such, the skilled artisan may identify and isolate genes of the endonuclease III family from other thermophilic bacteria. Suitable probes may be designed as degenerate nucleotide coding sequences for the following amino acid sequences which are highly conserved amongst the members of the endonuclease III family: SEQ1, PYVILITEILLRRTT; SEQ2, AILDLPGVGKYT; SEQ3, MVDANFVRVINR.

These degenerate oligonucleotides may be used as PCR primers to amplify portions of the gene from the chromosomal DNA of thermophilic bacteria by PCR. Such amplified PCR products may then be used to screen a library of the thermophilic bacterium. Positive clones would be sequenced and the coding sequence for the mismatch glycosylase cloned into an expression vector for protein production.

Additionally, the present invention can utilize a combination of nucleic acid repair enzymes. For example, a nucleic acid repair enzyme can be used in combination with a AP cleaving enzyme. Advantageously, mutY is used in combination with AP cleaving enzymes, such as DNA lyase or DNA AP endonuclease. Such a system of enzymes enhances the speed at which cleavage occurs.

As for the method step of partially digesting probe strands, partial digestion techniques are known to those skilled in the art. See Danna, supra. For the purposes of the instant description, a partial digestion denotes a situation where a nucleic acid does not effectuate complete cleavage of all probes stands in a reaction.

For example, where there are two points of mismatch on the probe-target hybrid, a partial digestion with a nucleic acid repair enzyme will not cleave every probe-target hybrid at both points of mismatch. Instead, 5 certain hybrids will be cleaved at the first point of mismatch, others will be cleaved at the second point of mismatch, and others still may be cleaved at both points.

A partial digestion can be established by various techniques known to those of skill in the art. For 10 example, a partial digestion is obtained by limiting the reaction time so that the nucleic acid repair enzyme does not cleave at all possible recognition sites within the probe oligonucleotide. Other methods for establishing a partial digestion include serial dilutions of a reaction 15 solution containing the enzyme or variation of the cation concentration in the solution.

The cleaved fragments resulting from the above-described partial digests are separated by size and visualized by methods known to those of skill in the art. 20 Such methods include gel electrophoresis and capillary electrophoresis as described above. The length of the cleaved fragments can be measured and, and thereby the specific nucleotide position of each point mutation determined, by comparing the probe fragments to labelled 25 DNA fragments of known size, as mentioned above.

In one embodiment of the present invention, the target polynucleotides and oligonucleotide probes have been amplified by techniques known to the skilled artisan, such as PCR, so that there is a sufficient 30 amount of probe fragments to be visualized, after the probe fragments have been separated by size in a visualizing medium.

In another embodiment of the present invention an oscillation reaction is employed to obviate or diminish 35 the need for amplified target polynucleotide. In particular, an oscillation reaction is created whereby a nucleic acid repair enzyme partially digests the oligonucleotide probe producing probe fragments which dissociate from the target polynucleotide at a



predetermined temperature. That is, the oligonucleotide probe is designed so that, at the predetermined temperature, the oligonucleotide fragments dissociate from the target polynucleotide after cleavage by nucleic acid repair enzyme. A cycle or oscillation reaction then occurs because the target polynucleotide hybridizes to another oligonucleotide probe, and the cleavage process is repeated.

As a consequence, a small number of target polynucleotides can be detected in a sample, since a single target polynucleotide catalyses the formation of a large number of oligonucleotide probe cleavage fragments. The oscillation reaction can detect from 10-100 target polynucleotide molecules in a sample. Theoretically, the oscillation reaction may detect as little as one target polynucleotide molecule in a sample.

To accommodate the oscillation reaction, a high concentration of oligonucleotide probe is utilized. In this regard, a suitable radiolabelled probe concentration is from 0.01 to 10 pmol. Other concentrations can be used depending on the desired length of autoradiograph exposure times.

One of skill in the art can refer to Duck et al., *BioTechniques* 9(2): 142 (1990), which refers to CPT a similar but less advantageous technique for amplifying probe.

In one embodiment, the oscillating reaction is performed isothermally, i.e., the predetermined temperature of dissociation is approximately the same as, i.e., within a few degrees of, the temperature of hybridization. In a preferred embodiment, this isothermal temperature is  $3N - 20^{\circ}\text{C}$ , here N is the length of the probe in base pairs. Within this working range the optimal temperature is determined empirically. Preferably, the reaction is performed with 0.01 to 10 pmol of labeled probe, in the presence of either synthetic target sequence or DNA purified from a sample source. This target DNA will ranges from 1 to  $10^{12}$  molecules.



In another embodiment, the oscillation reaction is not carried out isothermally, but instead results from temperature cycling. In this embodiment, the hybridization is effectuated at a temperature which is lower than the temperature of dissociation. In other words, after probe has hybridized to the target polynucleotide, the reaction temperature is raised to a predetermined temperature to effectuate the dissociation of the probe fragments from the target polynucleotide. The reaction is then cooled to allow non-cleaved probe to hybridize to the target polynucleotide, and the cycle is repeated. At this stage, more nucleic acid repair enzymes can be added if needed, to the extent that the original enzymes have lost activity due to the increase of temperature. In a preferred embodiment, the temperature is raised to between 85°C and 95°C for 1-2 minutes to dissociate the cleaved probe fragments from the target polynucleotide. The reaction is then slowly cooled to approximately 20°C to allow more non-cleaved probe to hybridize to the target polynucleotide, and the cycle is repeated.

In this preferred embodiment, an example of a preferred nucleic acid repair enzyme is thermophylic thymine DNA glycosylase, in particular, the enzyme is one encoded by the orf10 sequence of FIGURE 3. This enzyme will survive several cycles of exposure to 85°C for short periods.

In either of the above described isothermal or non-isothermal embodiments, hybridization can be facilitated by a helix destabilizing molecule. For instance, a helix destabilizing molecule can allow hybridization of a 20-mer synthetic oligonucleotide to target polynucleotide at 40°C.

By reducing the temperature necessary to achieve hybridization of oligonucleotide probe to target polynucleotide, helix destabilizing molecule can reduce the need for thermostable enzymes and expensive thermocyclers.

Exemplary helix-destabilizing molecules include \*I, herpes simplex virus-type I ICP8, nucleolin, and adenovirus DNA-binding protein. See Topal & Sinha, *J. Biol. Chem.* 258(20): 12274-79 (1983); Alberts & Frey, *Nature* 227: 1313-18 (1970); Hosoda & Moise, *J. Biol. Chem.* 253(20): 7547-55 (1978); Ghisolfi et al., *loc. cit.*, 267(5): 2955-59 (1992); Boehmer & Lehman, *J. Virol.* 67(2): 711-15 (1993); Zijderveld & van der Vleit, *J. Virol.* 68(2): 1158-64 (1994); Monaghan et al., *Nucleic Acids Research* 22(5): 742-48 (1994).

When facilitated by helix-destabilizing molecule, hybridization in accordance with the present invention can be effected with long synthetic oligonucleotides, without the use of thermostable enzymes or expensive thermocyclers. A "long" oligonucleotide in this context is greater than 25 nucleotides but preferably not greater than 100 nucleotides. Use of such long oligonucleotides affords the advantage of hybridizing to the target polynucleotide with increased specificity.

The presence of a helix-destabilizing molecule thus allows for the use of long synthetic oligonucleotides, without thermostable enzymes or expensive thermocyclers. The helix-destabilizing molecule allows for the dispensation of thermostable enzymes because it lowers the temperature necessary for hybridization.

The following examples merely illustrate the invention and, as such, are not to be considered as limiting the invention set forth in the claims.

#### EXAMPLE 1

#### PARTIAL DIGESTION DETECTION AND MAPPING OF MULTIPLE POINT MULTIPLE POINT MUTATIONS WITHIN A DNA SEQUENCE

The following synthetic oligonucleotides were synthesized using standard phosphoramidite chemistry, well known to those in the art.

35 5'-AAATGGAGTTATTCCAACAGATAAAGTGTGAATGGAATACTTAGTTAT  
3'-TTTACCTTAATAAGGTTGTATATTTTACCAATTACCTTATGAATTAATA

I                      II                      III                      IV

CTTGAATGACTAAAGTAGAATTAGA-3' (WT CS)  
GAACCTTAATGATTTTCAATTTAATCT-<sup>32</sup>P (MUT NCS)

V VI

5' - <sup>32</sup>P-labeled wild type noncoding strand (WT NCS) or  
5' - <sup>32</sup>P-mutated noncoding strand (MUT NCS) was annealed to  
the unlabeled wild type coding strand (WT CS).  
Mismatched bases in the NCS are underlined and numbered.  
Various amounts of thymine DNA glycosylase enzyme (Tdg),  
which removes the T at T/G mismatches or mutY enzyme,  
which cleaves the phosphodiester bond after the A at A/G  
mismatches were added and incubated for 1 hr at 55°C and  
37°C for the Tdg and mutY enzymes, respectively. In  
some cases, *E. coli* Endo IV was added to a set of Tdg  
enzyme tubes to facilitate phosphodiester bond cleavage  
at T/G mismatches where the Tdg enzyme has removed the T.  
At the end of the incubation, the DNA was electrophoresed  
on a 20% denaturing polyacrylamide gel. The gel was  
exposed to X ray film for various periods of time.

20 With a short exposure, the Tdg enzyme detected T/G mismatches I and III. Mismatches III, IV, and VI were detected following a longer exposure.

## EXAMPLE 2

# PARTIAL DIGESTION DETECTION AND MAPPING OF MULTIPLE POINT MULTIPLE POINT MUTATIONS WITHIN A DNA SEQUENCE

25           The following 75 base sequence of a mutated noncoding strand (NCS) was synthesized which generates two A/G and two T/G mismatches when hybridized to a wild type coding strand (CS):

30 5' - TCTAATTCTACTTTAGTAAATTCCAAGATAATTTAAGTATCCATT  
4 3  
AAACACTTTATTTGTTGGAATAACTCCATTT-3' Mut NCS  
2 1

The two A/G (numbers 2 and 4) and the two T/G mismatches (numbers 1 and 3), generated by hybridization of a labelled 75 base mutant probe (Mut NCS) to a wild-type target sequence (WT CS), were recognized by the DNA

mismatch repair enzymes mutY and Tdg enzyme, respectively, at dilutions of 1:100 and 1:10, respective temperatures of 55°C for Tdg and 37°C for mutY and a 1 hour incubation time. As controls, hybridization of a <sup>32</sup>P-labelled wild-type probe WT NCS to the same 75 base wild-type target WT CS polynucleotide failed to generate any mismatches and, therefore, no cleavage products were found.

### EXAMPLE 3

#### PARTIAL DIGESTION DETECTION AND MAPPING OF MULTIPLE POINT MULTIPLE POINT MUTATIONS WITHIN A DNA SEQUENCE COMBINED WITH AN OSCILLATION REACTION

The following synthetic oligonucleotides is synthesized using standard phosphoramidite chemistry, well known to those in the art.

5'-AAATGGAGTTATTCCAACAGATAAAGTGTTGAATGGAATACTTAGTTAT

3'-TTACCTTAATAAGGTTGTATATTTACCAATTACCTTATGAATTAATA

I

II

III

IV

CTTGGAATGACTAAAGTAGAATTAGA-3' (WT CS)

20 GAACCTTAATGATTTCATTTTAATCT-<sup>32</sup>P (MUT NCS)

V

VI

5'-<sup>32</sup>P-labeled wild type noncoding strand (WT NCS) or 5'-<sup>32</sup>P-mutated noncoding strand (MUT NCS) is annealed to the unlabeled wild type coding strand (WT CS). Mismatched bases in the NCS are underlined and numbered. Thymine DNA glycosylase enzyme (Tdg), which removes the T at T/G mismatches or mutY enzyme, which cleaves the phosphodiester bond after the A at A/G mismatches is added and incubated for 1 hr at 55°C and 37°C for the Tdg and mutY enzymes, respectively. In some cases, *E. coli* Endo IV is added to a set of Tdg enzyme tubes to facilitate phosphodiester bond cleavage at T/G mismatches where the Tdg enzyme has removed the T. At the end of the incubation, the tubes are incubated at 95°C for 2 minutes to denature the partial cleavage products from the WT CS. The temperature is slowly decreased to 20°C

to allow more  $^{32}\text{P}$ -labelled MUT NCS probe to anneal to the target WT CS. More Tdg enzyme or mutY enzyme are added to the appropriate tubes and incubation is continued for 1 hour at 55°C and 37°C, respectively. The cycle is then repeated. At the end of the final 1 hour incubation, loading buffer is added to the tubes. The DNA molecules are heat denatured and electrophoresed on a 20% denaturing polyacrylamide gel. The gel is exposed to X ray film, developed and analyzed.

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**EXAMPLE 4**  
**THERMOSTABLE ENZYME SYNTHESIS**

**Bacteria and Plasmids**

*Escherichia coli* JM109 is available from New England Biolabs of Beverly, MA and *Escherichia coli* BW415 is available from the laboratory of Dr. Richard P. Cunningham at the State University of New York, at Albany, Department of Biological Sciences. A similar strain suitable for this protocol is BW434 and it is available from the Coli Genetic Stock Center at Yale University School of Medicine, New Haven, CT.

BW415 $\lambda$ DE3 was made with a  $\lambda$ DE3 lysogenization kit from Novagen Inc. of Madison, WI. This integration allowed for the efficient expression of the T/G mismatch specific thymine-DNA glycosylase from a T7 RNA polymerase driven promoter in an endonuclease III deficient strain of *Escherichia coli*. The expression system was contained on plasmid pET14B from Novagen Inc.

Plasmid pUV2 containing the orf10 coding sequence is available from Dr. Jork Nolling, Wageningen Agricultural University of the Netherlands, Department of Microbiology, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands. The pUV2 plasmid contains a portion of pFV1, including the orf10 coding sequence, cloned into pUC19.

The methods for driving plasmid pFV1 are disclosed in Nolling et al., *Nuc. Acids Res.* 20(24): 6501 (1992). Plasmid pUC19 is available from Sigma Chemical Co., of St. Louis, MO.



## Preparation and Manipulation of DNA

Plasmid DNA was prepared from JME09 by a modified alkaline lysis method. The orf10 coding sequence from pUV2 was cloned into pUC19 via a DraI-EcoRI to HincII-EcoRI ligation using restriction endonucleases and ligase (from New England Bio Labs) according to suppliers' recommendations. The gene was PCR mutagenized using Taq Polymerase from Perkin Elmer Corp. to simultaneously change a TTG start codon to ATG, create a NcoI restriction site (5' GTG GGG CTG GAT TTC CAT GGA TGA TGC TAC TAA T3' and also a BamHI site (5' CGA CGG CCA GTG GAT CCA AGG GGG CTG ATG 3' outside the gene. These new restriction sites were used to clone orf10 into pET14B.

## Enzyme induction and Purification

This plasmid was transformed into *Escherichia coli* strain BW415λDE3 and selected from on ampicillin plates. Twenty four liters of cells were grown in Tryptone yeast (TY) broth supplemented with ampicillin at 37 C to an OD<sub>595</sub>=0.5, and induced with 1 mM IPTG for five hours. The cells were harvested by centrifugation at 17,000X g for 20 minutes to yield 100.82 grams of cell paste which was stored at -80C. The cell pellet was thawed, and suspended in 504 ml of 50mM Tris-HCl pH 8.0, 200 mM NaCl, 2.5mM EDTA 0.1 mM PMSF. The cell suspension was sonicated 5 X 3 minutes with a Branson sonifer, and then stirred on ice for one hour. The sonicate was centrifuged at 48,000X g for 20 minutes and the supernatant was retained. Five percent polyethelenimine was added to a final concentration of 0.1% and the suspension was stirred for 1 hour and centrifuged at 48,000X g to give a supernatant with a volume of 500 mls. The supernatant was dialyzed against 4 L of 50 mM KPO<sub>4</sub> pH 7.2 overnight.

The crude extract was loaded onto an 80 ml SP Sepharose Fast Flow (Pharmacia Biotech Inc. of Piscataway, NJ) column, washed with 100 ml of 50mM KPO<sub>4</sub> pH 7.2 and eluted with a 1 L gradient from 0 to 1M NaCl at a flow rate of 10 ml/min. The protein eluted at 0.6M NaCl as detected by 31 kD band visualized by SDS-PAGE,



and a characteristic yellow color in the fractions indicative of an Fe-S cluster of the protein. The fractions containing the protein were pooled and dialyzed against 50 mM KPO<sub>4</sub> pH 6.6, loaded onto a 5 ml DNA agarose (Pharmacia Biotech Inc.) column, washed with 10 ml of 50 mM KPO<sub>4</sub> and eluted with a 100ml gradient of 0-1 M NaCl at a rate of 1 ml/min. The protein eluted at 0.8M NaCl as determined by the criteria above. Fractions containing the protein were pooled and dialyzed overnight against 50 mM KPO<sub>4</sub> pH 6.6. The protein was loaded onto a 5ml SP high trap column (Pharmacia Biotech Inc.) and eluted with 1M NaCl to concentrate the protein. The extract was further concentrated to 1.5 mls with Centriprep 10 concentrators (Amicon Division, W.R. Grace & Co., of Danvers, MA). At this point the protein was a single band on an overloaded Coomassie stained gel and gave an A<sub>410</sub>/A<sub>280</sub> ratio of 0.295. This protein was active in our T-G mismatch assay. The pure protein was stored in 50% glycerol at -20 C.

**What Is Claimed Is:**

1. A method of detecting the presence of and determining the relative positions of at least two point mutations in target polynucleotides, comprising:

(a) hybridizing single-stranded oligonucleotide probes to target polynucleotides to form hybrid, double-stranded polynucleotides such that mismatches occur at the sites of said point mutations, wherein said probes are complementary to a non-mutated sequence of said target polynucleotides and are labelled at one end but not both ends, and wherein said target polynucleotides are not labelled;

(b) partially digesting the probe strands of said hybrid polynucleotides with a nucleic acid repair enzyme such that probe fragments of differing lengths are generated;

(c) separating said probe fragments by size in a medium suitable for visualizing the separated probe fragments; and then

(d) visualizing said separated probe fragments in said medium, whereby the presence and relative positions of said point mutations are determined.

2. The method of claim 1, comprising a further step of measuring the length of said separated probe fragments, thereby determining the specific nucleotide position of each point mutation.

3. The method of claim 1, wherein said partial digestion of step (b) is effected by a nucleic acid repair enzyme selected from the group consisting of mutY, T/G mismatch-specific nicking enzyme, human or yeast all-type enzyme, and deoxyinosine 3'-endonuclease from *E. coli*.

4. The method of claim 1, wherein said partial digestion of step (b) is effected by at least two nucleic acid repair enzyme selected from the group consisting of mutY, T/G mismatch-specific nicking enzyme, human or

yeast all-type enzyme, and deoxyinosine 3'-endonuclease from *E. coli*.

5. The method of claim 1, wherein said partial digestion of step (b) is effected by a nucleic acid repair enzyme comprising the combination of a glycosylase and DNA lyase or DNA AP endonuclease.

6. The method of claim 1, wherein said partial digestion of step (b) is effected by a thermostable nucleic acid repair enzyme.

7. The method of claim 1, wherein said thermostable nucleic acid repair enzyme is thymine DNA glycosylase.

8. The method of claim 1, wherein said thymine DNA glycosylase comprises an ORF10 protein encoded by a DNA sequence comprising the sequence of Figure 3.

9. The method of claim 1, wherein said labeled probe of step (a) is radiolabelled.

10. The method of claim 1, wherein said separating of step (c) is accomplished by gel electrophoresis.

11. The method of claim 1, wherein said target polynucleotides are cDNA sequences.

12. A method of detecting the presence of and determining the relative positions of at least two point mutations in a target polynucleotide, comprising:

(a) hybridizing a single-stranded oligonucleotide probe to a target polynucleotide to form a hybrid, double-stranded polynucleotide such that mismatches occur at the sites of said point mutations, wherein said probe is complementary to a non-mutated sequence of said target polynucleotide and is labelled at one end but not both

ends, and wherein said target polynucleotide is not labelled;

(b) partially digesting the probe strand of said hybrid polynucleotide with a nucleic acid repair enzyme producing oligonucleotide fragments, wherein said oligonucleotide probe is designed such that said oligonucleotide fragments dissociate from said target polynucleotide spontaneously at a predetermined temperature;

(c) repeating steps (a) and (b) such that probe fragments of differing lengths are generated;

(d) separating said probe fragments by size in a medium suitable for visualizing the separated probe fragments; and then

(e) visualizing said separated probe fragments in said medium, whereby the presence and relative positions of said point mutations are determined.

13. The method of claim 12, wherein said hybridization of step (a) is effectuated at a temperature which is approximately the same as said predetermined temperature of dissociation.

14. The method of claim 12, wherein said hybridization of step (a) is effectuated at a temperature which is lower than said predetermined temperature of dissociation.

15. The method of claim 12, comprising a further step of measuring the length of said separated probe fragments, thereby determining the specific nucleotide position of each point mutation.

16. The method of claim 12, wherein said partial digestion of step (b) is effected by a nucleic acid repair enzyme selected from the group consisting of mutY, T/G mismatch-specific nicking enzyme, human or yeast all-type enzyme, and deoxyinosine 3'-endonuclease from *E. coli*.

17. The method of claim 12, wherein said partial digestion of step (b) is effected by at least two nucleic acid repair enzyme selected from the group consisting of mutY, T/G mismatch-specific nicking enzyme, human or yeast all-type enzyme, and deoxyinosine 3'-endonuclease from *E. coli*.

18. The method of claim 12, wherein said partial digestion of step (b) is effected by a nucleic acid repair enzyme comprising the combination of a glycosylase and DNA lyase or DNA AP endonuclease.

19. The method of claim 12, wherein said partial digestion of step (b) is effected by a thermostable nucleic acid repair enzyme.

20. The method of claim 19, wherein said thermostable nucleic acid repair enzyme is thymine DNA glycosylase.

21. The method of claim 20, wherein said thymine DNA glycosylase comprises an ORF10 protein encoded by a DNA sequence comprising the sequence of Figure 3.

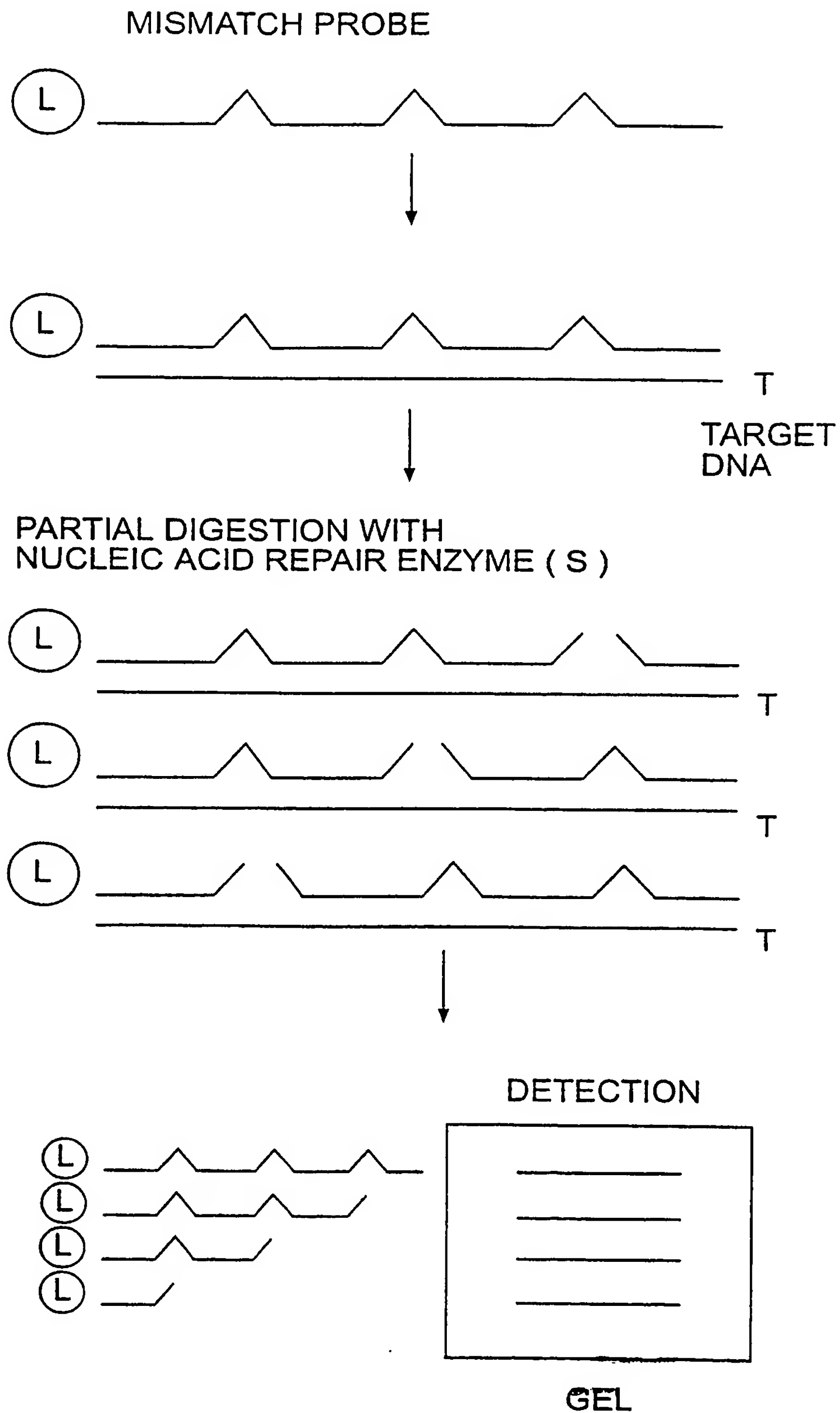
22. The method of claim 12, wherein said labeled probe of step (a) is radiolabelled.

23. The method of claim 12, wherein said separating of step (d) is accomplished by gel electrophoresis.

24. The method of claim 12, wherein said target polynucleotide is a cDNA sequence.

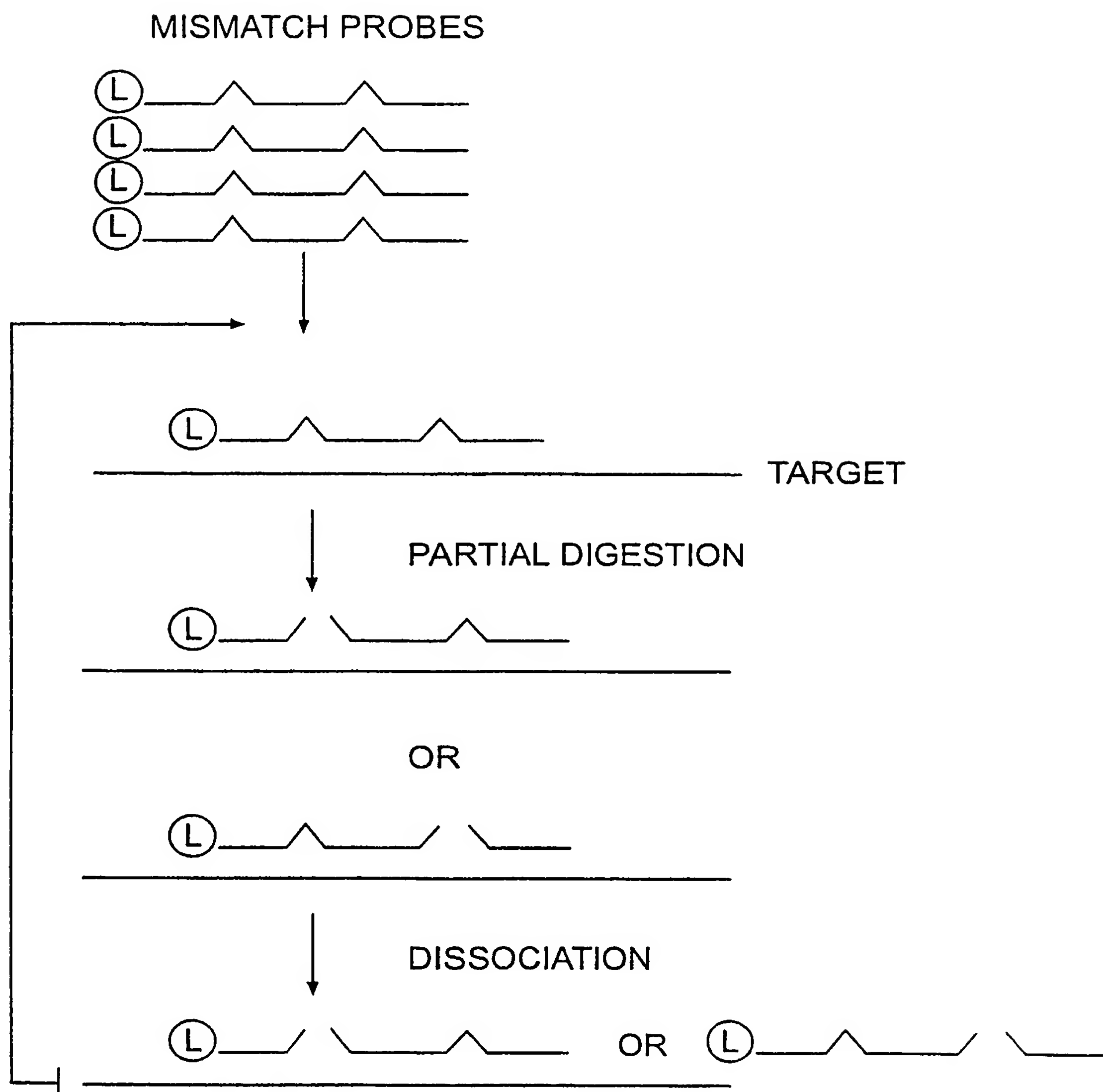
25. The method of claim 12, wherein a heat destabilization molecule is added to said hybridizing of step (a), such that the temperature of hybridization is decreased.

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**FIG. 1**





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**FIG. 2**

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**FIG. 3**

ATG	GAT	GAT	GCT	ACT	AAT	AAA	AAA	AGG	AAA	GTC	TTC	GTT	AGC	ACC	ATA	48
Met	Asp	Asp	Ala	Thr	Asn	Lys	Lys	Arg	Lys	Val	Phe	Val	Ser	Thr	Ile	
1				5					10					15		
CTT	ACG	TTT	TGG	AAT	ACA	GAT	AGG	CGC	GAC	TTT	CCT	TGG	AGG	CAT	ACG	96
Leu	Thr	Phe	Trp	Asn	Thr	Asp	Arg	Arg	Asp	Phe	Pro	Trp	Arg	His	Thr	
			20					25					30			
AGG	GAC	CCC	TAT	GTA	ATT	TTA	ATA	ACG	GAA	ATC	CTA	CTT	CGC	AGG	ACA	144
Arg	Asp	Pro	Tyr	Val	Ile	Leu	Ile	Thr	Glu	Ile	Leu	Leu	Arg	Arg	Thr	
		35					40					45				
ACT	GCG	GGG	CAT	GTT	AAA	AAG	ATA	TAT	GAC	AAG	TTT	TTT	GTT	AAG	TAC	192
Thr	Ala	Gly	His	Val	Lys	Lys	Ile	Tyr	Asp	Lys	Phe	Phe	Val	Lys	Tyr	
	50					55					60					
AAG	TGC	TTT	GAG	GAT	ATA	TTA	AAA	ACG	CCA	AAA	TCA	GAA	ATC	GCC	AAA	240
Lys	Cys	Phe	Glu	Asp	Ile	Leu	Lys	Thr	Pro	Lys	Ser	Glu	Ile	Ala	Lys	
65					70					75					80	
GAC	ATA	AAA	GAA	ATC	GGA	CTC	TCT	AAC	CAA	AGG	GCA	GAA	CAG	CTA	AAA	288
Asp	Ile	Lys	Glu	Ile	Gly	Leu	Ser	Asn	Gln	Arg	Ala	Glu	Gln	Leu	Lys	
				85					90					95		
GAA	CTG	GCA	AGG	GTC	GTC	ATA	AAT	GAT	TAT	GGG	GGC	AGA	GTG	CCC	CGA	336
Glu	Leu	Ala	Arg	Val	Val	Ile	Asn	Asp	Tyr	Gly	Gly	Arg	Val	Pro	Arg	
			100					105					110			
AAT	AGG	AAG	GCA	ATT	TTA	GAT	CTA	CCA	GGA	GTT	GGC	AAA	TAC	ACT	TGT	384
Asn	Arg	Lys	Ala	Ile	Leu	Asp	Leu	Pro	Gly	Val	Gly	Lys	Tyr	Thr	Cys	
		115					120					125				
GCT	GCA	GTT	ATG	TGT	TTG	GCA	TTT	GGC	AAA	AAA	GCC	GCT	ATG	GTC	GAT	432
Ala	Ala	Val	Met	Cys	Leu	Ala	Phe	Gly	Lys	Lys	Ala	Ala	Met	Val	Asp	
		130				135					140					
GCA	AAT	TTT	GTG	AGA	GTT	ATT	AAC	AGG	TAC	TTT	GGG	GGA	AGC	TAT	GAA	480
Ala	Asn	Phe	Val	Arg	Val	Ile	Asn	Arg	Tyr	Phe	Gly	Gly	Ser	Tyr	Glu	
					150					155					160	
AAC	CTG	AAC	TAC	AAC	CAC	AAG	GCC	CTG	TGG	GAA	CTT	GCG	GAG	ACC	CTT	528
Asn	Leu	Asn	Tyr	Asn	His	Lys	Ala	Leu	Trp	Glu	Leu	Ala	Glu	Thr	Leu	
				165					170					175		
GTA	CCT	GGC	GGA	AAG	TGC	AGG	GAC	TTT	AAC	CTT	GGT	TTA	ATG	GAC	TTT	576
Val	Pro	Gly	Gly	Lys	Cys	Arg	Asp	Phe	Asn	Leu	Gly	Leu	Met	Asp	Phe	
			180					185					190			
TCC	GCA	ATC	ATA	TGT	GCC	CCA	AGA	AAG	CCA	AAG	TGT	GAG	AAA	TGT	GGG	624
Ser	Ala	Ile	Ile	Cys	Ala	Pro	Arg	Lys	Pro	Lys	Cys	Glu	Lys	Cys	Gly	
		195					200					205				
ATG	AGC	AAA	CTC	TGT	AGC	TAC	TAT	GAG	AAG	TGT	AGT	ACT	TGA			666
Met	Ser	Lys	Leu	Cys	Ser	Tyr	Tyr	Glu	Lys	Cys	Ser	Thr	*			
	210					215					220					

# INTERNATIONAL SEARCH REPORT

In: International Application No  
PCT/US 98/06878

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C1201/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C120		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 40902 A (TREVIGEN INC) 19 December 1996 see the whole document ---	1-25
X	BENTLEY D R ET AL: "RAPID METHODS FOR DETECTION OF POLYMORPHIC MARKERS IN GENOMIC DNA" METHODS IN MOLECULAR BIOLOGY, vol. 9, 1 January 1991, pages 51-68, XP002004234 see the whole document ---	1-25
A	WO 97 12061 A (EPICENTRE TECHNOLOGIES CORP) 3 April 1997 see the whole document --- <div style="text-align: center;">-/--</div>	1-25
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
<b>Special categories of cited documents:</b> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center; font-size: 1.2em;">24 August 1998</div>		Date of mailing of the international search report  <div style="text-align: center; font-size: 1.2em;">28/08/1998</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx. 31 651 epo nl. Fax (+31-70) 340-3016		Authorized officer  <div style="text-align: center; font-size: 1.2em;">Hagenmaier, S</div>

# INTERNATIONAL SEARCH REPORT

In International Application No

PCT/US 98/06878

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 20233 A (UNIV MARYLAND) 14 October 1993 see the whole document ---	1-25
A	HSU I -C ET AL: "DETECTION OF DNA POINT MUTATIONS WITH DNA MISMATCH REPAIR ENZYMES" CARCINOGENESIS, vol. 15, no. 8, 1 August 1994, pages 1657-1662, XP000571680 see the whole document ---	1-25
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A	LU A -L ET AL: "DETECTION OF SINGLE DNA BASE MUTATIONS WITH MISMATCH REPAIR ENZYMES" GENOMICS, vol. 14, no. 2, 1 October 1992, pages 249-255, XP000571637 see the whole document ---	1-25
A	YAO M ET AL: "STRAND-SPECIFIC CLEAVAGE OF MISMATCH-CONTAINING DNA BY DEOXYINOSINE 3'ENDONUCLEASE FROM ESCHERICHIA COLI" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 50, 16 December 1994, pages 31390-31396, XP002018594 see the whole document ---	1-25
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Information on patent family members

International Application No

PCT/US 98/06878

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